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SOME OBSERVATIONS ON HEMOLYSIN- AND ACID-PRODUCTION BY STREPTOCOCCI *

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Although the hemolysin- and acid-production of streptococci from various sources have been studied by several investigators, we cannot overlook the fact that many important points pertaining to these characteristics remain to be decided more accurately. For instance, to what extent is the acid-production by streptococci in various carbohydrates to be regarded as a definite stable characteristic of certain strains, and what difference is there between a hemolytic halo and a greenish colorization of streptococcal colonies from the biochemical point of view?

The opinions of different investigators on these questions are not exactly coincident. Furthermore, there are various doubtful points with regard to the nature of the growth of streptococci, which should be determined more precisely. In the work included in this communication an attempt has been made to determine whether there is a contradictory relation between hemolysin- and acid-formation in glucose broth. To put the problem more clearly: On the one hand, it is a well known fact that hemolysis by streptococci is evidently hindered or decreased by the presence of glucose in the agar mediums; and, on the other hand, acid is produced in almost all strains in glucose fluid mediums. Looking at both phenomena together, we might be led to ask, if the hindrance of hemolysin-production in sugar mediums is caused by simultaneous acid-formations. Before entering directly into the subject the following preliminary observations were made, which have some bearing on the problem.

HEMOLYSIN TEST OF CULTURE FLUID AFTER INOCULATION

The streptococci which were used in this experiment were obtained from inflamed tonsils (1, 2, 3), erysipelas (4), and scarlatinal tonsils (5). They were cultivated, first, on the standard blood-agar plates; according to Becker,[1] distinctly hemolytic strains were selected and transplanted to ascites-broth (1:1) or horse-serum broth (1:1). These tubes were incubated for 8-15 hours at 37 C., then centrifugated, and the supernatant fluid used for the hemolysin test.

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[1] Jour. Infect. Dis., 1916, 19, p. 754.

TABLE 1

SCHEME A FOR HEMOLYSIN TEST

Number of Tube	Quantity of Culture Fluid, C.c.	Physiologic Salt Solution, C.c.	Defibrinated Isotonic Human Blood Suspension, C.c.	Percentage of Culture Mediums	Percentage of Blood
1	0.1	4.70	0.2	2	4
2	0.25	4.55	0.20	5	4
3	0.50	4.30	0.20	10	4
4	0.75	4.05	0.20	15	4
5	1.00	3.80	0.20	20	4
6	1.50	3.30	0.20	30	4
7	2.00	2.80	0.20	40	4
8	2.50	2.20	0.20	50	4
9	3.00	1.80	0.20	60	4
10	3.50	1.30	0.20	70	4
11	4.00	0.80	0.20	80	4
12	4.50	0.30	0.20	90	4
13	4.80	....	0.20	96	4

The comparison of the hemolysin-production of the 2 kinds of broth mixtures will be discussed later. The necessary quantity of fluid for this test was determined. The mixtures were placed at 37 C. for 2 hours, in the ice-box for 12 hours, and the result recorded. Five strains of hemolytic streptococci were examined by this method.

TABLE 2

THE HEMOLYTIC POWER OF THE STRAINS OF STREPTOCOCCI USED

No. of Strains	0.1 C.c.	0.25	0.5	0.75	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	4.8
1	—	—	—	+	+	+	+	+	+	+	+	+	+
2	—	—	—	+	+	+	+	+	+	+	+	+	+
3	—	±	±	+	+	+	+	+	+	+	+	+	+
4	—	±	+	+	+	+	+	+	+	+	+	+	+
5	—	±	+	+	+	+	+	+	+	+	+	+	+

After this a mixture of 0.5 c.c. and 1 c.c. of broth was used to test the hemolysis with different quantities of blood.

TABLE 3

SCHEME B_1 AND B_2 FOR HEMOLYSIN TEST

Scheme	Quantity of Culture Fluid, C.c.	Defibrinated Washed Human Blood, C.c.	Physiologic Salt Solution, C.c.	Percentage of Contained Blood
B_1	0.5	0.05	4.45	1
	0.5	0.10	4.40	2
	0.5	0.15	4.35	3
	0.5	0.20	4.30	4
	0.5	0.25	4.25	5
	0.5	0.30	4.20	6
	0.5	0.35	4.15	7
	0.5	0.40	4.10	8
B_2	1	0.05	3.95	1
	1	0.10	3.90	2
	1	0.15	3.85	3
	1	0.20	3.80	4
	1	0.25	3.75	5
	1	0.30	3.70	6
	1	0.35	3.65	7
	1	0.40	3.60	8

TABLE 4

HEMOLYSIN TESTS WITH DIFFERENT PERCENTAGES OF BLOOD *

Number of Strain	Percentage of Blood							
	1%	2%	3%	4%	5%	6%	7%	8%
1	± +	+ +	+ +	+ +	± +	∓ +	∓ ∓	∓ ∓
2	± +	+ +	+ +	+ +	∓ ∓	∓ +	∓ ∓	∓ ∓
3	± +	± +	+ +	+ +	− +	∓ ∓	∓ +	− +
4	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
5	± ±	+ +	+ +	+ +	+ ∓	∓ +	− +	− +

* In the tables the mark (±) signifies ambiguous result and the marks (∓) indicates incomplete hemolysis, that is, some unhemolyzed corpuscles remained at bottom.

Considering that the variations of hemolytic power may be easily noted at the border of positivity, I used 1 c.c. of the culture fluid, with 4% blood in the subsequent examinations, because if a larger amount of blood is used the slight decrease of hemolytic power might in some cases fail to attract attention.

Sensitivity of Erythrocytes of Different Animals to Hemolysin.—The blood of human beings, dogs, goats, sheep, rabbits, and guinea-pigs was used. The samples of blood were taken under sterile conditions, and the red corpuscles washed 3 times with physiologic salt solution, centrifugated, and mixed with salt solution equal in quantity to the serum taken off. The test was made according to Table 1.

TABLE 5

HEMOLYSIN TESTS WITH THE BLOOD OF DIFFERENT SPECIES

Number of Strain	Quantity of Culture Fluid, C.c.	Human Blood	Dog Blood	Goat Blood	Sheep Blood	Rabbit Blood	Guinea-pig Blood
3	0.1	−	−	−	−	−	−
	0.3	±	−	−	−	−	−
	0.5	±	±	±	−	±	±
	0.7	+	+	±	±	+	+
	1.0	+	+	+	±	+	+
4	0.1	−	−	−	−	−	−
	0.3	±	−	−	−	−	±
	0.5	+	±	±	±	±	+
	0.7	+	+	+	±	+	+
	1.0	+	+	+	+	+	+
5	0.1	−	−	−	−	−	−
	0.3	±	±	−	−	±	±
	0.5	+	±	±	±	+	+
	0.7	+	+	+	±	+	+
	1.0	+	+	+	+	+	+

Thus, in regard to sensitivity to hemolysin, the human blood corpuscle comes the first, and the corpuscles of guinea-pigs, rabbits, dogs, goats follow in the order given. The red corpuscle of sheep seems to have a slightly higher resistance to the hemolysin.

Filtrability of Hemolysin.—On the filtrability of hemolysin of streptococci the opinions of investigators have been divided; Some thought that the streptolysin was not filtrable; others claimed that it could be filtered. The recent publications on the subject record mostly positive results, although according to some authors (Hellens, etc.) the extent of filtrability varies.

In these experiments Berkefeld N filters and 15-hour cultures of streptococci in human or horse serum or ascites broth were used. The difference between the unfiltered culture and the filtrate with regard to hemolytic power is shown in Table 6.

TABLE 6

COMPARISON OF HEMOLYTIC POWER OF UNFILTERED CULTURE FLUIDS

Number of Strain	Culture Fluids		0.1	0.25	0.5
2	Human serum + broth	Unfiltered	—	—	—
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	—	—
		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	±
		Filtrate	—	—	—
4	Human serum + broth	Unfiltered	—	±	+
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	—	+
		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	±
		Filtrate	—	—	—
5	Human serum + broth	Unfiltered	—	—	+
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	±	+
		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	+
		Filtrate	—	—	—

As shown, the filtrate in all cases undoubtedly possesses less hemolytic power than the unfiltered culture. Whether this difference is exclusively due to the living streptococci in the unfiltered culture is not easily decided, because in some cases the beginning of the hemolytic process in the unfiltered culture fluid could be observed even very soon after incubation, too soon indeed for any action of bacteria, while the filtrate did not cause any such phenomenon after a longer period.

For obtaining the hemolysin the human serum mixed with plain broth in equal parts seems to have a slight superiority over other mixtures. the ascites fluid and horse serum have about the same power for this purpose, yet in the case of Strain 4, the former showed an earlier production of the hemolysin than the latter, while the latter finally showed the greater production if cultivated for a longer period than the former. Moreover, we cannot overlook a certain predilection of certain strains of streptococci for particular mediums as regards hemolysin-formation.

Time of Hemolysin-Production.—Strongly hemolytic strains of streptococci sometimes clearly show the hemolytic halo on blood agar after 1 or 2 hours in the incubator (usually 5-8 hours), and the same results may be observed in fluid cultures. In most instances hemolysin begins to develop after 3 hours' incubation at 37 C., and usually increases until the 15th-18th hour. Some strains do not show much difference in hemolytic power between 18 and 24 hours, while others show an evident reduction of hemolysin after 18 or 24 hours' cultivation. The streptolysin survives usually only a short time in fluid mediums. This has been noted previously by many. Although it is sometimes demonstrated 3-5 or 7 days after cultivation, this is rather exceptional, and generally the degree of hemolytic power is rapidly reduced after 24 hours. Even in the refrigerator the hemolysin shows a marked decrease hour by hour (Table 7).

The figures in Table 7 indicate the hemolytic power expressed by the reciprocal value of the culture fluids used.

TABLE 6—Continued
COMPARISON OF HEMOLYTIC POWER OF UNFILTERED CULTURE FLUIDS

0.75	1.0	1.5	2.0	2.5	3.0	3.5	4.0
+ —	+ ±	+ +	+ +	+ +	+ ±	+ +	+ +
± ±	+ ±	+ +	+ +	+ +	+ ±	+ +	+ +
+ —	+ +	+ +	+ ±	+ ±	+ +	+ +	+ +
+ —	+ ±	+ +	+ ±	+ +	+ +	+ +	+ +
+ —	+ ±	+ +	+ +	+ ±	+ +	+ +	+ +
+ —	+ ±	+ —	+ ±	+ ±	+ +	+ +	+ +
+ —	+ ±	+ —	+ +	+ +	+ +	+ +	+ +
+ —	+ ±	+ —	+ —	+ +	+ ±	+ +	+ +
+ —	+ ±	+ —	+ ±	+ ±	+ +	+ +	+ +

TABLE 7
DURATION OF CULTIVATION IN RELATION TO PRODUCTION OF HEMOLYSIN

Number of Strain	Hours of Incubation										
	1	3	5	8	10	15	18	24	36	48	72
2	—	—	0.5	1	1	1	1	0.5	—	—	—
4	—	0.4	0.66	1	2	2	2	1.3	0.33	0.25	—
5	—	—	0.33	1	1	2	2	1	0.33	—	—

TABLE 8
FERMENTATION TEST OF THE STRAIN OF STREPTOCOCCI USED

No. of Strain	Plain Broth	Glucose	Inulin	Lactose	Maltose	Mannite	Neutral Red	Raffinose	Saccharose	Salicin
1	—	+	—	+	+	—	—	—	+	±
2	—	+	—	+	+	—	—	+	+	+
3	—	+	—	—	+	—	—	—	—	±
4	—	+	—	+	+	—	—	—	+	+
5	—	+	—	+	+	—	—	—	+	+

Acid Formation by Streptococci.—The strains of streptococci used in these experiments varied in fermentative power, as shown in Table 8.

To decide whether acid is produced or not, the culture mediums were titrated before and after cultivation, and the difference of acidity in both titrations was regarded as the degree of acid production.

Time of Cultivation in Relation to Acid-Formation.—Using 1% glucose broth for cultures, the results in Table 9 were obtained.

TABLE 9

TIME OF CULTIVATION IN RELATION TO ACID-FORMATION

Number of Strain	Hours of Incubation										
	3	5	8	10	15	18	24	36	48	72	96
1	—	—	—	2.5	3.2	3.2	5.0	5.0	5.0	4.8	5.0
2	—	0.1	0.1	0.3	1.5	2.8	4.1	4.0	4.1	4.1	0
3	—	—	0.3	0.8	2.5	3.0	5.2	5.2	5.0	5.2	5.2
4	—	—	1.0	1.2	3.0	4.4	5.6	5.6	5.6	0	0
5	0.5	0.6	0.5	1.0	2.4	3.8	4.8	4.8	4.8	0	0

The acid-formation was not visible before 3 hours. Within 5-10 hours, when hemolysin-production seems usually to take place, the production of acid appeared to be very slight; after this, however, the degree of acid-formation was rapidly increased, and it reached the climax in 24-48 hours. After 48 hours the amount of acid produced was not increased, a fact which is not difficult to understand, since it is well known that streptococci usually cease to grow in fluid mediums after 48 hours.

Percentage of Glucose Used in the Broth in Proportion to Acid-Formation.—The percentage of glucose used in the broth is of some importance in the production of acid: 1-3% of glucose showed in many cases the same high formation of acid. In excess of 5% of glucose, acid-formation seemed to be markedly reduced, although the growth of streptococci was not distinctly hindered by the higher content of glucose (Table 10).

TABLE 10

PERCENTAGE OF GLUCOSE IN PROPORTION TO ACID-FORMATION

No. of Strain	Medium	0.25	0.5%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	15%	20%	25%
2	Broth................	2.0	4.0	4.0	4.0	3.8	3.2	2.5	—	—	—	—	—	—	—	—
	Broth + horse serum	2.8	3.5	4.1	4.1	4.1	3.8	3.2	3.2	1.0	0.8	—	0.2	—	—	—
	Broth + ascites fluid	2.5	3.8	4.2	4.1	4.0	4.1	3.8	3.0	0.8	0.8	—	0.1	—	—	—
4	Broth................	2.0	2.4	5.4	5.4	5.0	3.4	3.5	—	—	2.0	1.8	0.5	—	—	—
	Broth + horse serum	3.0	5.2	5.6	5.6	5.6	3.9	5.2	4.0	4.2	2.8	3.0	—	—	—	—
	Broth + ascites fluid	2.8	5.0	5.5	5.3	5.5	4.8	5.0	3.5	3.0	—	1.0	—	—	—	—
5	Broth................	3.0	4.5	4.8	4.8	4.2	3.8	2.8	3.0	2.8	2.0	1.8	—	—	—	—
	Broth + horse serum	3.5	4.8	4.8	4.8	4.5	4.3	4.0	4.0	3.8	2.0	2.3	1.8	0.5	—	—
	Broth + ascites fluid	3.2	4.8	4.8	4.8	4.3	4.1	4.5	4.1	1.8	1.8	1.5	0.5	—	—	—

If we take into consideration the difference between the 0.5% and 3% glucose broth, the 3 kinds of broth did not show much difference in acid-production by cultivation of streptococci. The fact that the broths with horse serum or ascites fluid indicated acid-formation even in a higher content of glucose than the broth without such mixture might be because they are more suitable mediums for streptococci than the unmixed glucose broth, although the streptococci would grow little less in the latter than in the former. In other words, in the glucose broth to which horse serum or ascites fluid had been added the streptococci could exhibit and maintain their vitability in a higher content of glucose than in the simple glucose broth.

Hemolysis Shown by the Acid Produced.—The acid produced in glucose broth by streptococci was strongly hemolytic for the washed erythrocytes of all the animals examined.

The test was made as follows: The broth cultures of varying contents of glucose were incubated for 24 hours, and then heated to 60 C. for 30 minutes. Five c.c. of each broth culture of the different percentages of glucose were taken and neutralized with 1/20 normal sodium hydrate to the point of acidity of the original broth before incubation. Thus the degree of the acid produced was determined. The other portions were used in the hemolysin test in graduated quantities.

TABLE 11

HEMOLYTIC POWER OF ACID PRODUCED

Number of Strain	Percentage of Glucose	Quantity of Culture Fluid, C.c.					Titer of Acid Produced
		0.1	0.3	0.5	0.7	1.0	
4	0.5	—	—	±	+	+	2.4
	1.0	—	+	+	+	+	5.4
	2	±	+	+	+	+	5.4
	3	—	±	+	+	+	5.0
	4	—	—	+	+	+	3.4
	5	—	—	+	+	+	3.5

The same test was made with Strains 2 and 5, the results being about the same.

Hemolysin-Production in Glucose Broth.—In this experiment I added 1/20 normal sodium hydrate to culture mediums with different amounts of glucose, in order to reduce the acidity to the acid degree of plain broth, and after cultivation for 15 hours the necessary amount of normal sodium hydrate was once more added to neutralize the acid produced. The hemolysin test was then tried, according to Table 1.

TABLE 12

HEMOLYSIN-PRODUCTION IN GLUCOSE BROTH

Number of Srtain	Plain Broth	Glucose Broth, %						
		0.25	0.5	1	2	3	4	5
1	+ (0.75)	—	—	—	—	—	—	—
2	+(0.75)	+(2.5)	+(2.5)	—	—	—	—	—
3	+(0.75)	+(2.0)	+(2.0)	+(2.5)	—	—	—	—
4	+(0.5)	+(1.5)	+(1.5)	+(2.0)	+(2.0)	+(2.5)	—	—
5	+(0.5)	—	—	—	—	—	—	—

The figures within the brackets indicate the quantities of culture fluid necessary to give positive results in the hemolytic test.

I found in this test that distinct hemolysin-production occurred only in plain broth, seldom in a weak content of glucose, and very rarely in 3% of glucose as in the case of Strain 4, while the titration of acid produced in that same percentage in dicated 5.

Longevity of Streptococci in Broth with Various Amounts of Glucose.—Experiments have been made to ascertain what influence the various percentages of glucose in broth may have on the power of growth of streptococci in relation to survival.

After 24 hours in the incubator, the tubes which contained the higher content of glucose (6 or 7%) scarcely showed turbidity at the bottom; however, the streptococci could be found in stained films of fluid at the bottom of the tubes.

The form and shape of the bacteria were not much changed, even in broth which contained 9 or 10% of glucose. With a higher percentage of glucose some of the streptococci were distinctly agglutinated, but the others still remained in the normal chain form.

Transplants on blood-agar slants after various periods at room temperature gave the results shown in Table 13.

TABLE 13

SURVIVAL OF STREPTOCOCCI IN GLUCOSE BROTH OF DIFFERENT STRENGTHS

Number of Strain	Weeks	Plain Broth	Glucose Broth, %														
			0.25	0.5	1	2	3	4	5	6	7	8	9	10	15	20	25
2	1	—	+	+	+	+	+	+	+	—	+	+	—	+	+	—	—
	3	—	—	+	+	+	+	—	+	—	—	—	—	—	—	—	—
	5	—	—	+	+	+	+	—	—	—	—	—	—	—	—	—	—
	10	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
4	1	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
	3	—	+	+	+	+	+	+	—	—	—	+	—	—	—	—	—
	5	—	—	+	+	—	+	—	—	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	1	+	+	+	+	+	—	+	+	+	—	+	+	—	+	—	—
	3	+	+	+	+	+	—	+	+	—	—	—	—	—	—	—	—
	5	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

As shown, the glucose content of 0.5-2% was most favorable for survival of streptococci. With an increased percentage of glucose the streptococci could live for a certain time only.

DISCUSSION

Hemolysin is found in 1% glucose broth cultures, while the content of acid produced in the same culture reaches a comparatively high amount. At all events, if the hemolysin-production is unmistakably reduced or totally hindered in fluid mediums containing glucose it does not seem to be true that the hemolysin-formation is checked or destroyed by the acid produced, because in some cases the hemolysin was still found even in a high degree of acid-formation; in other cases the former was not found at all when there was very slight or no formation of the acid in glucose broth. The probable explanation is that, although the hemolysin-production is an important characteristic for the classification of certain kinds of streptococci, it needs an adequate culture medium, and can be easily hindered by an inadequate amount of a certain ingredient of the mediums and not by the produced acid. To repeat, the hemolysin does not seem to appear at all in a medium inadequate for its production regardless of whether the acid is produced or not.

The formation of hemolysin reaches its climax in 8-15 hours, and after about 18-24 hours is rather rapidly diminished in quantity; while the production of acid is usually slow in the first 5-10 hours, and most marked in 24-48 hours after incubation. The degree of acid-formation is highest between a 0.5 and 2 percentage of glucose content, and decreases in intensity above 5%. At any rate, if the content of glucose exceeds a certain limit, the power of acid-formation of streptococci is undoubtedly hindered, although the ability of streptococci to live and to grow is not yet completely checked by such a high content of glucose. Thus from my experiments it seems probable that the ability to produce hemolysin is an attribute which is not always concomitant with the vitality of streptococci, and that it is easily checked by the addition of a certain quantity of glucose. In comparison, the acid-formation goes hand in hand with the vitality of streptococci, although the parallel is not constant, that is, if in a high content of glucose the streptococci lose, more or less, the ability of acid-formation they may yet retain vitality without much change.

Taking into consideration the results of all observations, the following conclusions seem warranted:

Hemolysin-production by streptococci in glucose-containing fluid mediums does not stand with regard to time in reciprocal relation to acid-formation.

The ability of streptococci to produce hemolysin in glucose-containing mediums is checked by the glucose, and can not be considered hindered or destroyed by simultaneous acid-formation.

The ability to form acid in its turn does not altogether coincide with the ability of streptococci to live. It can be reduced or totally hindered in a higher content of glucose in fluid mediums, although the ability of streptococci to live still remains in some degree.

The production of hemolysin begins and ends earlier than the production of acid. The former can be demonstrated in many cases after 3-5 hours of cultivation, and reaches its climax in 15-18 hours, while the latter seldom appears so early (that is, within 3-5 hours), and its climax occurs between 24-48 hours.

Although the ability to produce hemolysin and acid by streptococci is important for their differentiation, yet they are not in concordance with the power of streptococci to live. In other words, the vitality of streptococci can exist intact, even if the power to form hemolysin and acid is nullified in certain culture mediums.